PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No.	:	10/677,701
Applicant	:	Victor V. Levenson *et al.*
Filed	:	October 2, 2003
Title	:	METHYLATION PROFILE OF BREAST CANCER (as amended)
TC/A.U.	:	1634
Examiner	:	Jeanine Anne Goldberg
Docket No.	:	5369-00011

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop: Amendment
Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Victor V. Levenson, M.D., Ph.D., being duly warned, hereby declare and state that:

1. I am a co-inventor of the invention disclosed and presently claimed in the above-captioned patent application.

2. The attached document entitled "Grant Proposal" (EXHIBIT A) describes experiments that were performed utilizing methods disclosed and presently claimed in the above-captioned patent application. The Grant Proposal was submitted to the Susan Koman Foundation and was not funded.

3. The Grant Proposal discloses experiments in which breast cancer was diagnosed or characterized in patients by detecting methylation in DAPK1 and additional genes that together were utilized as a composite "biomarker." (*See* Grant Proposal at page 4.)

4. As discussed in the Grant Proposal, plasma samples from twenty-nine (29) normal patients or plasma samples from twenty-nine (29) patients with ductal carcinoma *in situ* (DCIS) were obtained. Genomic DNA was isolated from the samples, and the methylation status of DAPK and additional genes including FAS, MCTS1, CDKN2A, PAX5, PGK1, RPL15, THBS, TNFSF11, and VHL was assessed utilizing the methylation assay disclosed in the above-captioned patent application. Approximately 89.3% of DCIS samples were found to exhibit methylation in the DAPK1 gene while only 51.9% of normal samples were found to exhibit methylation in the DAPK gene. Utilizing statistical methods disclosed in the Grant Proposal, the biomarker comprising DAPK1 and additional genes was shown to identify DCIS with approximately 84% sensitivity and approximately 80% specificity. (*See* Grant Proposal at page 4, Table 4 and at pages 7-8, under heading "Statistical analysis".)

5. Also as discussed in the Grant Proposal, plasma samples from three (3) healthy patients or plasma samples from three (3) patients with atypical ductal hyperplasia (ADH) also were obtained. Genomic DNA was isolated from the samples and the methylation status of DAPK and additional genes was assessed utilizing the methylation assay disclosed in the above-captioned patent application. (*See* Grant Proposal at page 4, Table 5, and accompanying text.) Approximately 37.5% of ADH samples were found to exhibit methylation in the DAPK1 gene, while no healthy samples were found to exhibit methylation in the DAPK gene (0%).

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Date: **April 22, 2008**

By **/Victor Levenson/**
Victor V. Levenson

GRANT PROPOSAL

(Exhibit A)

Blood-based Biomarkers of Breast Cancer Risk

In this project we will develop and validate a blood-based biomarker for detection and diagnosis of atypical ductal hyperplasia (ADH). We will use a novel technique of methylation analysis to detect ADH-specific changes of methylation in cell-free circulating DNA from blood. As a result we will produce a clinically relevant biomarker, which can be used for screening of women at risk for breast cancer development. The diagnostic biomarker developed in this project will be ready for a prospective clinical trial.

• **Background.** Despite efforts in fight against breast cancer, each year the disease causes nearly 40,000 deaths in the US. Ductal carcinoma is the most common type of breast cancer, accounting for nearly 75% of all cases.[1] Early detection of the tumor, when it is still small and well-contained, remains the most efficient way to reduce cancer-related mortality. Currently detection relies on screening by mammography, which is limited by tissue density (100% sensitivity in fatty versus 47% - in dense breasts), stage of tumor (81% for invasive ductal carcinomas (IDC) versus 55% for ductal carcinomas *in situ*, DCIS), age, hormonal status, and other issues.[2] As a result, screening benefits mostly women older than 40 with late stage tumors.[3] This situation is clearly unacceptable and calls for development of alternative detection techniques in order to identify an earlier lesion, atypical ductal hyperplasia (ADH),[4, 5] and recognize women at risk for breast cancer development. The diagnostic criteria of ADH are imperfect, and rely on the absence of certain features of DCIS,[6] size of the lesion, and histologic and cytologic criteria.[7] Nonetheless, association of ADH with greater risk of invasive ductal carcinoma is firmly established,[8-10] so identification of ADH in a screening assay will be clinically significant. In this project we will develop a biomarker for detection and diagnosis of ADH.

Biomarkers. A screening test has to measure tumor-related factors in an observer-independent assay. Detection based on such biomarkers is not prone to errors from poorly controlled circumstances, and allows standardization of the test procedure.

► *An observer-independent biomarker can eliminate many problems of a screening test associated with variability of subjective readout; such a biomarker will improve accuracy of disease detection.*

Ideal biomarker. There are several well-established principles for biomarker development. An ideal biomarker should be specific for a particular disease and undetectable under physiological conditions; the specimen for the assay should be collected in a minimally invasive and inexpensive manner; the assay should be easy, reproducible, rapid, and inexpensive,[11] and dynamic changes of biomarker should reflect disease progression (Fig. 1). Known genetic markers (e.g. mutations) do not reflect variability at either cellular or clinical level and remain constant (no dynamic range). Protein marker levels are tightly linked to functional activity of the cell, and can widely fluctuate (dynamic range unrelated to disease). As a result, genetic markers can predict risk but will not detect changes specific for the onset of cancer, while protein levels change due to unrelated factors, either masking the disease or producing false-positive results.

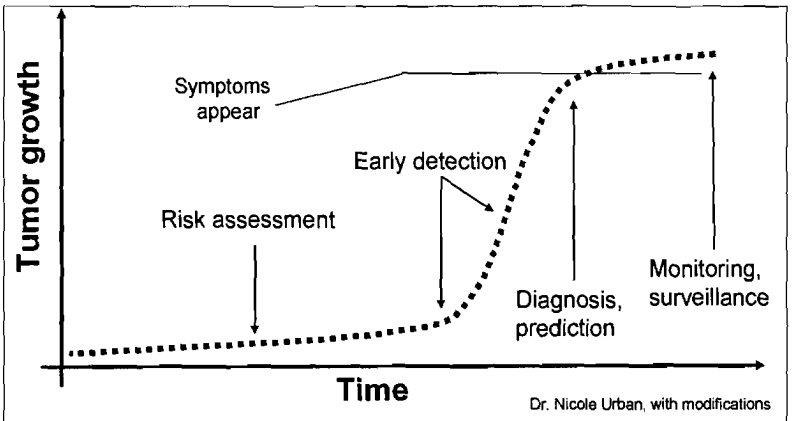

Fig. 1. Different biomarkers and their role in evaluation of tumor growth.

► *The ideal biomarker should reflect functional change (e.g., from low to high risk, or from high risk but disease-free status to overt but early disease) but should not be influenced by unrelated events.*

Correlative and mechanistic biomarkers. Any feature that is linked to a disease can be used as a biomarker. A simple association with a clinical symptom is sufficient to use a feature as a biomarker even when its basis is unknown (correlative biomarkers; e.g. fever as a classic biomarker of infection). Alternatively, biomarkers can emerge from analysis of disease mechanisms (mechanistic biomarkers). Once the natural history

of the disease is known, the possibilities are well-defined, and a search for mechanistic biomarkers can be very efficient. In complex diseases such as cancer poorly understood process of tumor initiation does not allow targeted testing and initially requires correlative biomarkers for risk assessment and early detection.

At first correlative biomarkers may have no obvious links to the known pathology of a disease: amplification of a certain DNA fragment in drug resistant cells[12] was initially established as a correlative marker, and the mechanistic value of this correlation was revealed later (discovery of multidrug resistance gene MDR1 in the amplified fragment[13]). Similarly, Ki-67 antibodies were developed and their value for tumor assessment was established before even the composition of the Ki-67 antigen was known;[14] it is revealing that Ki-67 function even now is enigmatic.[14] A systematic search for disease-specific correlative biomarkers requires comparison of multiple elements in disease and healthy tissues; further analysis of these biomarkers can reveal previously unsuspected features and new disease mechanisms, which can result in new treatments.

►*For complex diseases with poorly understood pathogenesis there is insufficient information for development of mechanistic biomarkers. In contrast, correlative biomarkers satisfy the immediate medical needs, and provide the groundwork for mechanistic understanding of the disease.*

Biomarkers for breast cancer detection and risk assessment. While multiple biomarkers exist for breast cancer monitoring,[15] biomarkers for early detection remain a challenge. HER2/neu amplification has been suggested for risk assessment in asymptomatic women,[16, 17] but the assay requires tissue biopsy, which will make it unsuitable for many women. Analysis of biomarkers in nipple aspirate fluid or in ductal lavage is too labor-intensive, expensive, and unreliable,[18] leaving blood as the medium of choice. Tumor cells,[19] tumor-specific antigens and autoantigens,[20] tumor RNA and DNA[21, 22] can be recovered from blood, and have been tried as breast cancer biomarkers (lipids,[23] polyamines,[24] proteins,[25] RNA,[21] and DNA[22]), although none has yet emerged as a biomarker for breast cancer.

DNA methylation as a biomarker. DNA based biomarkers have a significant advantage because DNA can be amplified by PCR. Epigenetic change (DNA methylation) is a stable modification, which is linked to gene expression and reflects the cell's function.[26] Methylation is precisely located in cytosines within the cytosine-guanosine dinucleotides (CpG) and CpG islands of gene promoters,[27] and the first methylation-based biomarker for breast cancer was recently reported (unfortunately, its accuracy was low even for invasive cancer).[28]

►*Although potential of DNA methylation for development of breast cancer biomarkers is recognized, there are no biomarkers even for early detection. No biomarkers for risk assessment are available.*

Composite methylation biomarker. A search for methylation biomarkers would be uncomplicated if a certain region was always methylated in breast cancer. However, there is only a certain probability of methylation, and no specific pathology is invariably associated with methylation of a unique gene. This situation can be improved if multiple genes are analyzed to create a methylation profile.[29] These profiles for patients and healthy controls can then be compared to select genes with the most pronounced differences in methylation. Such differentially methylated genes will be combined to form a composite correlative biomarker. Successful selection of appropriate genes depends on their number in the initial methylation profile: the more genes analyzed, the better are the chances of finding highly significant differentially methylated genes, which will form a successful composite biomarker. This approach will be used for our project.

► *Multiple genes analyzed for methylation in each sample create the sample's methylation profile; genes that are consistently selected as differentially methylated in methylation profiles of healthy individuals and patients with ADH will generate a composite biomarker for ADH.*

Techniques for methylation detection can be chemical or biological: bisulfite chemically converts unmethylated cytosines into uracils, leaving methylated cytosines intact;[30] alternatively, DNA can be treated with methylation-sensitive restriction enzymes, which digest only unmethylated but not methylated DNA.[31] Bisulfite conversion is a harsh treatment, which destroys 85-95% of input DNA,[32] while milder procedures lead to incomplete conversion and ambiguous results.[33] Bisulfite modification is combined with different detection techniques, e.g. sequencing, methylation-specific PCR[34] and its derivatives. All methods except sequencing have limited resolution and evaluate methylation only in some cytosines in each fragment. In addition, in a

heterogeneous clinical sample only some DNA fragments of each kind may be methylated, and positive methylation readout tends to ignore similar but unmethylated fragments. To avoid this error, additional testing is required, so two different reactions have to be performed, one for methylated target and the other for unmethylated.[34] If the sample is indeed heterogeneous and both methylated and unmethylated sequences are present, the overall result can be ambiguous.[34]

Alternatively, detection of methylation can use different activity of certain restriction enzymes on methylated and unmethylated DNA. Such enzymes leave DNA undigested if a cytosine within the recognition site is methylated, so only non-methylated templates are destroyed while methylated templates are preserved and can be detected.[35] If all restriction sites within the region are methylated, the whole region is scored as methylated; a single unmethylated site will lead to destruction of the template's integrity, and the region will be scored as unmethylated. To make comparison between different fragments possible, a similar number of sites in each fragment is analyzed. In homogeneous systems, detection of methylation is relatively simple.[36] In heterogeneous clinical samples the threshold to define methylated and unmethylated fragments is established to standardize the readout (see **Study Design**).

► *Bisulfite methods of DNA methylation analysis cause degradation of the major part of the sample, depend on chemical changes in DNA sequence, and may produce ambiguous results. In contrast, methylation-sensitive restriction enzyme-based techniques do not inflict DNA damage or changes to DNA sequence, and are designed to produce unambiguous data.*

Special features of clinical samples. Cinical samples are always limited and heterogeneous, so differences in analytical techniques are especially important. Bisulfite treatment destroys over 80% of input DNA,[32] triggering concerns about specific sequences that are preferentially destroyed. Even if there is no preference, limited amount of DNA from a clinical sample is further diminished by bisulfite treatment.

The bulk of cytosines is located outside of CpG dinucleotides, so they are not methylated. All of them are converted to uracils by bisulfite, which causes major changes in DNA sequence. As a result, unanticipated effects can emerge, e.g. differences in PCR efficiency for sense and antisense strands of DNA.[37]

Some bisulfite-based techniques allow quantitative measurement of methylation at specific sites, which is important for homogeneous samples but becomes a liability for heterogeneous samples. For example, methylation of stratifin increases in ductal carcinoma cells with tumor development, but in stromal cell the same gene is completely methylated in normal breast and in tumor.[38] As a result, the same tumor sample will give different results depending on the number of stromal cells in the test sample. Restriction enzyme-based techniques avoid quantitative assessment, so differences in stromal cells will not affect the readout, and stratifin will be scored as "methylated".

► *DNA destruction in bisulfite reaction is a concern for clinical samples, which are limited and heterogeneous (a specific fraction of DNA may be more sensitive to degradation). Quantitative analysis of methylation by bisulfite-based techniques may also complicate the interpretation because specimen heterogeneity rather than disease-related changes may be responsible for quantitative differences. Restriction enzyme-based techniques are not prone to these problems.*

Plasma as the source of tumor-specific DNA has been used to analyze tumor-specific mutations (e.g.[39]) and methylation (e.g.[28]). Plasma DNA is found in healthy subjects;[40] in cancer patients its concentration is much higher.[41] Considering that risk assessment will target asymptomatic women, our assay has been intentionally developed for use with plasma, which will allow repeated sampling and will not cause undue discomfort associated with tissue biopsy. It is important to emphasize that cell-free circulating DNA in plasma should be considered a heterogeneous clinical sample raising all the concerns mentioned in the previous section.

• *Tumor-specific DNA from plasma allows analysis of tumor-specific methylation in samples collected with a minimally invasive procedure. Cell-free DNA from plasma should be considered a heterogeneous clinical sample.*

Assay for methylation detection has been developed in our laboratory[36] and successfully used with clinical samples [42]. Methylation status of 56 genes (a profile) is determined for each sample (Fig. 2A). The profiles are processed to find differentially methylated genes in control and disease samples (Fig. 2B), and these genes form

a composite biomarker. A custom-designed array is used for signal detection.

Table 1. Breast cancer detection in tissues.

Predicted status		Actual status: Cancer	Actual status: Normal	
	pCancer	**0.7239**	0.2526	1
	pNormal	0.2761	**0.7474**	
		DCIS	Normal	
	pDCIS	**0.7048**	0.1869	2
	pNormal	0.2952	**0.8131**	
		ADH	Normal	
	pADH	**0.8750**	0.0501	3
	pNormal	0.1250	**0.9499**	
		IDC	Normal	
	pIDC	**0.7056**	0.2686	4
	pNormal	0.2944	**0.7314**	

Restriction digestion provides at least a 100-fold reduction in the unmethylated template after digestion.[36]

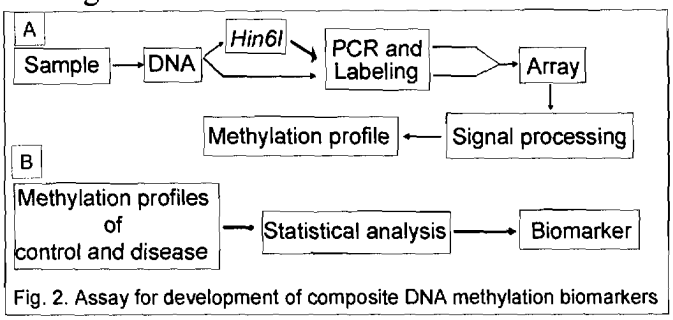

Fig. 2. Assay for development of composite DNA methylation biomarkers

Tissues of breast, ovarian, and brain cancer, and plasma DNA from ovarian, lung, pancreatic, and prostate cancer were analyzed. For breast cancer (tissues) the accuracy of cancer detection ranges between 70 and 95% (Table 1), and is the best for ADH lesions (set 3), where core biopsies were analyzed. Heterogeneity of the tissue sections (DCIS and IDC samples) probably conceals many differences in methylation that are revealed in a more homogeneous material from core biopsies (ADH samples). Indeed, the composite biomarker for DCIS and IDC has 10 genes, while the biomarker for ADH has 9 additional genes (Table 2). The set of genes is almost identical for DCIS and IDC biomarkers, and genes for ADH are present in the profiles for DCIS and IDC. These similarities indicate that the biomarkers identify common biological features of breast cancer and can recognize it at the stage of ADH.

Table 2. Biomarker genes in tissues.

	ADH	DCIS	IDC	Cancer
EP300	x	x	x	x
MGMT	x	x	x	x
TP73	x	x		x
PGR (distal pr)	x		x	x
THBS1	x		x	x
PYCARD (TMS1)	x	x	x	x
PRKCDBP (SRBC)	x	x		
FABP3 (MDGI)			x	x
MSH2		x		x
HIC1		x	x	x
BRCA1	x			
TES	x			
NR3C1 (GR)	x			
ICAM1	x			
DAPK1	x			
TNFSF11 (RANKL)	x			
DNAJC15 (MCJ)	x			
CDH1	x			
CASP8	x			
RPL15	x			
PGK1	x			

Table 3. Genes of the biomarker and their methylation in plasma of DCIS patients and healthy controls.

Gene	DCIS	Normal
DAPK1	89.3%	51.9%
FAS	82.8%	33.3%
MCTS1	66.7%	28.6%
CDKN2A	74.1%	35.3%
PAX5	75.0%	40.0%
PGK1	78.6%	37.5%
RPL15	53.8%	13.0%
THBS	82.1%	36.8%
TNFSF11	72.7%	30.0%
VHL	91.3%	25.0%

Table 4. DCIS detection in plasma.

	DCIS	Normal
pDCIS	84.48%	19.87%
pNormal	15.52%	80.13%

Table 5. Differences in methylation detected in plasma DNA.

Gene	Methylated %	
	Healthy	ADH
BRCA1	75	37.5
DAPK1	0	37.5
ESR1	87.5	50
FAS	12.5	50
MDR1	12.5	50
MGMT	0	50
MLH1	25	50
MUC2	87.5	50
p15INK4B	50	87.5
p16INK4A	12.5	62.5
p73	25	50
PGK1	25	50
RASSF1A	100	37.5
RB1	87.5	50
THBS1	12.5	37.5
TNSF11	12.5	50
UPA	75	37.5
VHL	12.5	37.5

Tissue biomarkers have less clinical value than biomarkers from blood, so we compared profiles of plasma DNA for patients diagnosed with DCIS and healthy controls. Ten genes showed significant differences in methylation (Table 3) and were selected for the composite biomarker. We tested this biomarker with 25 rounds of 5-fold cross-validation and registered sensitivity and specificity of DCIS detection (statistical procedures are described in the **Study Design**). Results indicated that the biomarker identified DCIS with 84% sensitivity and 80% specificity (intersections of predicted and actual sample status) with type II error at 16% and type I error at 20% (Table 4).

For this proposal we compared methylation profiles of cell-free circulating DNA from ADH patients and healthy women in a small set of samples (Table 5; eight samples per group).While this set is by no means conclusive, it shows that differences in DNA methylation can be detected in plasma of ADH cases and healthy controls. Our previous experience indicates that genes with a 20% methylation difference in the trial set almost always become components of the composite biomarker, suggesting that 18 genes can contribute to the ADH biomarker.

• **Hypothesis and objective.**

DNA methylation is different in normal and cancerous tissues; cancer-specific DNA can be detected in bloodstream (cell-free plasma DNA); a composite biomarker has been developed for tissue-based breast cancer detection; a similar biomarker has high sensitivity and specificity for detection of DCIS using cell-free plasma DNA; multiple differences have been detected in methylation of plasma DNA from patients with ADH and healthy controls. *Based on these findings we hypothesize that a composite biomarker based on methylation profiling of cell-free plasma DNA can be developed for patients with ADH.* Our objective is development and validation of such biomarker.

• **Specific Aims.**

♦ ***Aim 1:*** to develop a methylation-based composite biomarker for detection of atypical ductal hyperplasia.

We will analyze methylation of 56 genes in each sample of circulating plasma DNA of three groups of subjects: women with ADH found in biopsy; women whose biopsy results revealed only benign disease; healthy women. All groups will have a similar age distribution for participants; there will be 35 independent samples (collected from 35 different patients) in each group. We will then identify genes differentially methylated in ADH patients and control groups and select informative genes for the biomarker. The sensitivity and the specificity of the biomarker will be determined using 25 rounds of 5-fold-cross validation.

♦ ***Aim 2:*** to validate performance of this composite biomarker for detection of atypical ductal hyperplasia using blinded specimens.

The composite biomarker for ADH developed in Aim 1 will be tested in a separate blinded set of 60 independent specimens. The set will contain 20 samples from each of the three groups, and the accuracy (specificity and sensitivity) of ADH detection will be determined.

♦ ***Aim 3:*** to analyze performance of this composite detection biomarker for diagnosis of atypical ductal hyperplasia using a separate set of blinded specimens collected at a breast clinic from 100 women. About 25 of these specimens are expected to have ADH.

We will test the composite detection biomarker developed in Aim 1, using 100 blinded specimens collected at a breast clinic. These specimens will contain samples from healthy women and women with different types of cancer and benign breast disease including microcalcifications, so the diagnostic potential of the developed biomarker will be established.

► *This approach will determine (Aim 1) and validate the accuracy of ADH detection (Aim 2), and the accuracy of ADH diagnosis in a set of samples representing different diseases of the breast (Aim 3).*

• **Study Design.**

Overview. In this study we will design and verify the composite biomarker using the training group of 105 open-label samples. The developed biomarker will be validated in a blinded group of 60 specimens of ADH and controls to assess its performance for ADH detection. Then the biomarker's performance will be evaluated in a blinded group of 100 samples from women with *different diseases of the breast* to evaluate its performance as a diagnostic biomarker (expected frequency of ADH in women with microcalcifications is 31%).[43] This strategy follows the standards of biomarker development,[11] and emphasizes ADH detection and diagnosis as the primary goal. Evaluation of the biomarker with blinded samples completes phases 2 and 3 of biomarker development.

Patients. Plasma specimens will be collected from women with small lesions (mammography - less than 2 mm[44]) prior to biopsy at OSF Saint Anthony Center for Cancer Care, Rockford, IL. Samples are then stratified as ADH, DCIS, LCIS, IDC or benign based on the pathology report of the excised lesion. This report is then confirmed by Dr. Barbara Susnik (Northwestern University, Department of Pathology), who will review all samples for consistency. Plasma collection prior to biopsy will prevent any changes in the methylation profile related to anesthesia or biopsy itself. While different descriptions of ADH have been suggested [6, 10, 45], in the most current definition ADH is a uniform population of regularly arranged small round, cuboidal or polygonal hyperchromatic cells; nuclei are evenly distributed, and only single small nucleoli are seen; mitoses, especially abnormal, are infrequent.[44] "Healthy" controls are defined as women without neoplastic or chronic

inflammatory disease. To account for age-related methylation changes[46] all groups will have comparable age distribution. Control groups will contain specimens from women whose biopsy results are negative for ADH and contain only benign lesions, and specimens from healthy women, collected separately. There is no data for race-related differences, but this option will be considered in the statistical analysis. The project has been reviewed and approved by the Institutional Review Board (IRB) of Northwestern University and of the OSF Saint Anthony Center for Cancer Care. Collection protocol ensures that specimens will include plasma from women with LCIS, DCIS, IDC, and benign breast disease including microcalcifications; in this latter group 31% of patients are expected to have ADH.[43]

Assay. Analytical and statistical techniques have been tested with over 300 specimens of DNA from tissues (breast, ovarian, and brain cancer) and plasma (breast, ovarian, prostate, lung, pancreatic, and colon cancer). Plasma-based biomarkers have been designed for ovarian cancer (Melnikov, submitted) and for DCIS (Melnikov, in preparation). To assess methylation, purified plasma DNA is divided into two parts – one is treated with the methylation-sensitive restriction enzyme *Hin6I*, and the other is untreated. Methylated fragments are preserved, while the unmethylated template DNA is destroyed and cannot serve as a template for PCR. Both digested and control DNAs are used for nested PCR with gene-specific primers; aminoallyl-dUTP is added for the second round of amplification. The aminoallyl groups of incorporated dUTPs are then coupled to reactive Cy5 or Cy3 dyes, creating fluorescently labeled products. One dye is used for PCR products from undigested control DNA, another – for PCR products from *Hin6I*-digested DNA. Both labeled products are hybridized to a custom-designed microarray with probes for amplified fragments. Each microarray has three identical subarrays to follow consistency of the signal. Fluorescence of both fluorophores in every spot is quantified, and the Cy5/Cy3 ratio is calculated.

A Cy5/Cy3 ratio specific for completely methylated DNA in each spot is determined and used to define methylation status for each fragment. Several spots provide quality control: empty spots determine background, spots for unmethylated targets serve to control efficiency of digestion, and spots with probes for non-human DNA define nonspecific binding.

Statistical analysis compares methylation profiles in cases and controls to select the most differentially methylated genes. These genes are then analyzed as a group, and the classification accuracy is established. Data analysis is described below.

Methods.

Collection of blood follows the standard procedure with EDTA-containing BD Vacutainer tubes. Tubes are centrifuged at 1,100×*g* for 10 min. Supernatant is collected, placed into another tube, and centrifuged again. Final plasma is collected and frozen at -70°C.

Purification and quantitation of cell-free circulating DNA. Plasma (100 µl) is mixed with 2^x Proteinase K buffer (100 mM NaCl, 10 mM TrisHCl, pH8.0, 10 mM EDTA, 0.5% SDS) and incubated at 55°C for 6 hr with Proteinase K (1 mg/ml). DNA is purified with DNAzol (MRC, Cincinnati, OH): DNA is mixed with 10 vol of DNAzol, and DNA is precipitated by 0.5 vol of 100% ethanol, washed with 80% ethanol, and dissolved in 10 mM Tris pH7.8, 0.5 mM EDTA. Concentration is determined using Hoechst 33248 and DyNAQuant 2000 (Hoefer, GE Healthcare, Piscataway, NJ).

Digestion with methylation-sensitive restriction enzyme Hin6I (Fermentas, Hanover, MD; recognition site GCGC) is done in 100 µl at 37°C. Two ng of DNA is incubated with 40 U of the enzyme. The second aliquot is incubated without the enzyme, processed side-by-side with digested DNA as a control, and only fragments with a signal from control DNA are scored.

PCR amplification: 400 pg of digested and control DNAs are used for the first round of nested PCR. KlenTaq1 (DNA PolTech, St. Louis, MO) is used at 20 U per 50 µl reaction. PCR is done for 25 cycles (95°C; 45sec – 62°C; 1min – 72°C; 1min). QIAquick PCR Purification Kit (Qiagen, Valencia, CA) is used to purify PCR products. For the second PCR 400 pg of combined products are used; PCR contains a mix of aminoallyl-

dUTP (Biotium, Hayward, CA) and dTTP (3:1), and is done as before for 20 cycles. PCR products are purified with QIAquick PCR Purification Kit, eluted and combined.

Coupling aminoallyl-labeled PCR products to Cy dyes. Purified products of the second PCR are dried and dissolved in 5 µl of 200 mM $NaHCO_3$ (pH 9.0). Cy3 or Cy5 in DMSO are added and mixed. Labeling continues for 2 hrs at room temperature in the dark, and the labeled products precipitated with ethanol.

Table 6. Genes for analysis of methylation.

Apoptosis	Cell cycle	Signal transduction	Transcription factors	Other
DAPK	CYCD2	ESR1(A,B)	HIC1	MDGI
RANKL	RB1	GR	BRCA1	RIZ
APAF1	p15INK4B	PR (A, B)	SOCS1	TES
TMS1	p16INK4A	RASSF1A	FHIT	uPA
CASP8	p21WAF1	EDNRB	PAX5	MCT1
FAS	p27Kip1	S100A2	VHL	HIN1
	p57Kip2	SRBC		EP300
	p73	SYK		MUC2
	SFN			MCJ
				CALC
Angiogenesis	**Differentiation**	**Drug resistance**	**Invasion**	**Repair**
THBS1	MYOD1	MDR1	CDH1	MLH1
	RARbeta2	GSTP	GPC3	MSH2
		RFC	ICAM1	MGMT

Development of the array. All the genes for the array (Table 6) have been shown to be methylated in breast cancer. Oligonucleotide arrays (with 50-60 mer probes) are custom designed by Microarrays, Inc (Nashville, TN). Three sets of control probes are present – transcribed regions from *A. thaliana* (definitive negative control, heterologous); transcribed regions of human α-tubulin, β-actin and GAPDH (definitive negative controls, homologous); promoters of β-actin, phosphoglycerate kinase and ribosomal protein L15 (conditional homologous negative control). Oligonucleotides contain an amino group and a six-carbon spacer at the 5'-end. They are spotted on aminosilane-modified glass slide in triplicate, so each slide contains three identical subarrays.

Hybridization. The slides are pre-hybridized in rotating tubes at 42°C in 5xSSC, 0.1% SDS, 1% BSA, denatured DNA is added, the coverslip is sealed, and the slides are hybridized for 18 hr at 42°C. After hybridization the slides are washed at 42°C in 1xSSC, 0.1%SDS and in 0.1xSSC, 0.1%SDS, and dried.

Detection of the signal. ScanArray™ 4000XL (Perkin-Elmer, Wellesley, MA) is used to record the signal. Signal is acquired by the EasyScan™ using the Adaptive Circle algorithm.

Normalization of the signal is designed for a 'directed array', in which all Cy5/Cy3 ratios should be greater than or equal to 1. Complete methylation of a fragment should produce a Cy5/Cy3 ratio of 1 if fluorophors' performance is identical. Since fluorophores are different, a coefficient for normalization of the Cy5/Cy3 ratios is established using a "self-self" technique (Fig. 3);[47] a single PCR product is separated into two parts, each is labeled with a different fluorophor, mixed, and used for hybridization. In this mixture Cy5- and Cy3-labeled fragments are equally represented imitating completely methylated fragments for each spot, so fluorophor-related discrepancy in detection can be adjusted with "self-self"-derived coefficients (Standard Methylation Calls or SMCs).

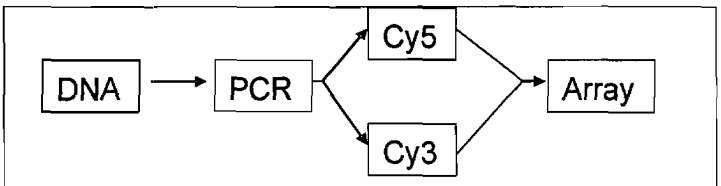

Fig. 3. Experimental approach to determine methylation threshold.

Statistical analysis. The first step involves non-specific filtering to remove unreliable data and retain only informative genes. "Unreliable" is determined as a gene where signal from undigested DNA is less than 3^x background. "Informative gene" is determined as a gene that gives interpretable methylation call in at least 80% of all samples. After filtering, Cy5/Cy3 ratio for completely methylated DNA (SMC) is used to dichotomize data for each gene into "methylated" (M; ratio ≤ SMC), and "unmethylated" (UM; ratio ≥ 1.1 times SMC); data between these thresholds is scored as "unassigned" (UA; SMC < ratio < 1.1 times SMC) and removed. Potential components of the biomarker are then selected by the chi-square test of association and Fisher's Exact Test with a 0.10 significance level. *P*-value correction for multiple comparisons is not essential, since differential methylation of individual genes is just an intermediate result. Significance at a nominal alpha level of 10% effectively serves as a filter for informative gene selection. The naïve Bayes algorithm for pattern recognition

tasks[48] is used to identify genes for the composite biomarker. Misclassification errors are determined by twenty five rounds of stratified five-fold cross-validation.[49] For each iteration the data are randomly divided into five groups with four groups assigned to a learning set, and the fifth group left as a testing set. The naïve Bayes algorithm is applied to the learning set to choose contributing genes and establish classification parameters, which are then used with the test set to determine its accuracy. The process is repeated five times so that each group is used as the test set once. The sets are then combined, and another iteration begins. Previous experience indicates that at least 5-10 genes will be found as differentially methylated in each cross-validation step. The final biomarker will contain genes that are consistently chosen in the cross-validation steps. The biomarker will be considered successful if cross-validation achieves at least 85% sensitivity and at least 80% specificity for ADH. Several alternate classification methods will be assessed to determine the robustness of the conclusions to the choice of statistical algorithms. Logistic regression and partial least squares will be employed as the alternate classification methods.[50]

To detect potential confounding effects of clinical variables (e.g. age, race), we will use both supervised and unsupervised methods. We will perform standard unsupervised hierarchical clustering[51] to explore potential subgroups within the patient set and examine the relationship of such subgroups to relevant clinical factors. The supervised analysis will be based on the classification methods described above (e.g. Naïve Bayes) with clinical factors added as potential classifiers. Both main effects (effects of clinical factors on disease status) and interaction effects (different disease/methylation relationship within different clinical subgroups) will be explored.

Specimens. Three sets of samples will be analyzed – a learning set to develop the biomarker as described above (35 open-label samples from ADH patients, 35 samples from healthy controls and 35 – from controls with benign diseases); the first validation set to assess the biomarker performance for ADH detection (20 samples from patients with ADH, 20 healthy and 20 benign controls), and the second validation set to assess the biomarker performance for ADH diagnosis (100 blinded samples from women diagnosed with different breast diseases). All validation samples will be blinded by the principle investigator before submitting the data for biostatistical analysis.

The sample size calculations are made with the following assumptions:

1) Aim 1. For biomarker selection, we assume markers will be methylated at a rate of approximately 50% in cases and 25% in controls, and an initial biomarker filter of $p<0.1$ (two sided Fisher's Exact). A sample size of 35 per group will give 70% power on an individual marker basis to detect a difference in the methylation rate. Based on our pilot data, this power should be sufficient to identify enough candidate biomarkers for development of the classification algorithm.
2) Aim 2, First Validation set. In order to demonstrate that the sensitivity and specificity is significantly superior to 50% (two sided Fisher's exact p-value <0.05), a sample size of 40 controls and 20 cases will give 90% power under the assumption of 80% specificity and 85% sensitivity.
3) Aim 3, Second validation set. Since we do not know *a priori* what the case/control distribution in the second validation cohort will be, the power analysis is based on overall accuracy. Assuming that the overall diagnostic accuracy is 80%, a sample size of 100 subjects will be required to have the 95% confidence interval for overall accuracy to have a width of +- 7.5%

Evaluation of results. Progress of the project will be evaluated by the selection of genes for the biomarker, and by the misclassification rates after cross-validation (see *Statistical Analysis*). Based on pilot data, we expect that the test will have 85% sensitivity and 80% specificity. The project will be evaluated at each of the three stages (development, validation of detection, validation of diagnosis) using the same criteria. Accuracy of detection and diagnosis by the methylation assay will be determined as the match to the pathology report. Even in case when the accuracy is below projected, the results will still be an important step indicating that the methylation-based diagnostic assay is possible if adjustments to the approach are made (see below).

Two independent validation sets will be used to confirm the sensitivity and specificity of the biomarker. Our data show sensitivity of 87.5% and specificity of 94.99% for ADH detection in tissues (Table 2); results of DCIS detection in plasma (Table 4) indicate that 85% sensitivity and 80% specificity are realistic.

The first validation set contains 20 ADH and 40 control samples; this validation will assess the biomarker performance for ADH *detection*. In the second validation set the fraction of ADH will not be known *a priori*, and the set will contain different types of breast pathology. This set will provide a realistic assessment of the power of the methylation biomarker to *diagnose* ADH, i.e. to identify ADH amongst a number of different breast diseases.

Validation of methylation results is done by bisulfite sequencing for selected genes in five randomly chosen specimens from each group. Bisulfite sequencing is done as before[36] with several modifications for limited amounts of starting template. DNA is purified,[52] and treated with 9M bisulfite for 40 min at 70°C to reduce DNA degradation.[53] PCR primers for selected regions are designed with MethPrimer.[54] Amplified regions are cloned into pGEM-T-Easy and 20 individual clones are sequenced from both ends using M13 sequencing primers.[36]

► *All techniques for this assay have been tested with multiple specimens of DNA from tissues, and from plasma of cancer patients and healthy controls. The assay has been extensively controlled and validated, so no significant problems are expected.*

Potential problems and solutions.

Technical problems. Difficulties in morphological discrimination between DCIS and ADH may result in different assessment of the same sample by different pathologists; such samples will be considered as borderline DCIS and replaced. The analytical space of 56 genes may not be sufficient to find genes for stage-specific diagnosis, and profiles for ADH and DCIS may be substantially similar; the accuracy of the biomarker will then be determined in comparison of "disease" vs. control groups, and appropriate restrictions will be described for the clinical use of the biomarker.

Biological problems. ADH, DCIS, IDC may represent different morphological forms of the same disease; then morphological differences will not be reflected on molecular level, and molecular differentiation between these forms will be impossible. If that is the case, the project will define and validate the biomarker for *the earliest possible molecular detection* of breast cancer, and as such will have the same impact on clinical practice. Finally, profiles of benign diseases may be too similar to the ADH-DCIS-IDC profile; this result will indicate that benign diseases contain some elements of malignant growth. To discriminate between these possibilities we consider a follow-up study of women with benign diseases.

► *None of these problems will affect the course of the study, but rather will be the result of the project. Clinical application will not be affected because the biomarker will still identify the earliest possible stage of the disease. Our understanding of breast cancer will be advanced whether epigenetic differences between ADH and DCIS are detected or not.*

There is a possibility, which is not very likely according to the preliminary data, that the projected accuracy of 85% sensitivity and 80% specificity will not be achieved using the current set of genes. In that case we will select no more than five fragments with the most significant differences between ADH and control samples (assessed by the *p*-value) as the basis of the composite biomarker. Additional genes will be selected from literature and tested in batches; the most differentially methylated genes (assessed by the *p*-value) will be added to the composite biomarker.

Finally, reduced accuracy of detection may reflect heterogeneity within the ADH group – it is possible that two subgroups of ADH patients have similar morphological changes, and only one of them has higher risk of breast cancer. In this case we may expect to detect heterogeneity in molecular profiles, which will reduce accuracy of ADH detection. To test this possibility we will perform unsupervised hierarchical clustering as described above using centered Pearson correlation to measure the distance between the two groups. The threshold value separating the two clusters will be selected to be higher than the similarity values within each cluster. Biomarker testing will then be done separately for each group, and their pathological characteristics will be compared. At this time, it is impossible to estimate the likelihood of this situation, but we are prepared to re-evaluate our results should experimental data point in this direction.

• **Clinical impact.** A blood-based detection of ADH will make a considerable impact on breast cancer by providing a minimally invasive molecular test for the earliest stage of the disease. Selection of a subset of genes

for testing will provide an opportunity to develop a relatively inexpensive screening assay that can be offered for population-wide screening. The impact will be especially significant for younger women with dense breasts, because in this group mammography is ineffective as a screening tool and is not recommended by the American Cancer Society[55]. Identification of ADH patients as a high-risk group will allow better targeted interventions, including preventive regimens. Even if molecular profiles of ADH and DCIS turn out to be indistinguishable, the project will create a clinically appropriate biomarker for the earliest possible detection of breast cancer. By the end of the project the test for detection of ADH will be developed and validated. The test will allow repeated sampling and will be ready for validation in a prospective trial, which will involve screening of asymptomatic women to correlate the test's performance and results of mammography/biopsy.

• **Innovation.** To our knowledge this is the first test that evaluates methylation status of 56 genes using cell-free DNA from 0.2 ml of plasma. This test is also the first test to achieve 84.5% sensitivity and 80% specificity for DCIS detection. While each step of the assay is not novel by itself, the end result has been achieved for the first time. The assay can be adjusted to include additional genes for improved sensitivity and specificity. A statistical algorithm for tumor detection has been developed specifically for this assay. The assay is intended as a tool for assessment of personal risk of breast cancer development, and as a method for individualized cancer monitoring. It is important to notice that the assay serves to identify components of the biomarker rather than to perform the test itself; multiple techniques can be used to analyze a limited number of genes once those informative for the disease have been selected.

Collaborative Efforts and Future Directions

This project involves collaboration between the PI (development of the assay) and the breast cancer oncologist (Dr. Hoskins, OSF Saint Anthony Center for Cancer Care, Rockford, IL). Once the assay is developed and validated, additional funds will be sought to initiate a clinical trial at this clinic. The goals of the trial will be to correlate performance of the developed methylation test with mammography/biopsy techniques in women presenting at the OSF Saint Anthony Center for Cancer Care.

The biomarker developed in this project will be ready for clinical application, which will be done using a simple PCR-based detection. This shift in technology depends on the task - while the microarray-based technique is valuable as a tool for screening multiple genes in each sample, it is too sophisticated for routine use in a clinical laboratory. Moreover, its use will be unnecessary since the informative genes will already be identified, and a PCR-based technique will be the most appropriate clinical test for a small number of informative methylation markers selected in this project.

Dissemination Plan.

The results of the project will be published in three installments: development of the composite biomarker and its performance in an open set (target - *Breast Cancer Research*); performance of the biomarker with the blinded set of samples from breast cancer patients and healthy controls (target – *Clinical Cancer Research*); and performance of the biomarker in the blinded set of samples from breast care clinic (target – *Journal of Clinical Investigation*). Results will also be presented at the San Antonio Breast Cancer Symposia, and AACR meetings.

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